

Transactivation by hepatitis B virus X protein is promiscuous and dependent on mitogen-activated cellular serine/threonine kinases

(AP-1/Raf-1/protein kinase C)

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ABSTRACT The X protein of hepatitis B virus (HBV-X) can act as a transactivator of transcription but its mechanism of action remains obscure. We have analyzed HBV-X transactivation in several cell types using 13 unrelated viral and cellular promoters and found that transactivation is more or less apparent in most cell types and is promiscuous and unrelated to specific sequence motifs within the target promoters. In general, though, HBV-X appears to act on enhancer elements since HBV-X had no effect on a minimal promoter, whereas HBV-X was able to transactivate after insertion of an AP-1 minienhancer. Several lines of evidence exclude the possibility that HBV-X interacts directly with the AP-1 enhancer or its binding proteins and suggest that the proximal target of HBV-X is peripheral to the transcription complex. This hypothesis is supported by the observation that inhibition of serine/threonine kinases, which regulate AP-1 activity (phorbol ester down-regulation or staurosporine inhibition of protein kinase C and a dominant negative mutant of Raf-1), blocked the ability of HBV-X to transactivate without affecting basal promoter activity. Furthermore, basal transcription from the AP-1-dependent promoter was increased by overexpression of protein kinase C and Raf-1 but HBV-X was unable to further stimulate, indicating that these kinases act subsequently to HBV-X. These data suggest that transactivation by HBV-X is an indirect result of the activation of cellular serine/threonine kinases including protein kinase C and Raf-1. This mode of action implies that HBV-X may affect other cellular processes, besides transcription, that are regulated by these kinases.

Hepatitis B virus (HBV) is an important cause of both acute and chronic liver disease in humans, and HBV carriers are predisposed to developing hepatocellular carcinoma. The virus has a compact genome of only 3.2 kb containing four open reading frames: pre-S/S encoding the surface antigens, pre-C/C encoding c-antigen and e-antigen core proteins, P encoding the viral reverse transcriptase, and X. The HBV-X gene encodes a protein of 154 aa. The sequence bears little resemblance to other known proteins but is highly conserved among subtypes of the human virus and viral homologues that infect ground squirrels and woodchucks (1, 2). The function of HBV-X is unknown but several studies have shown that HBV-X can transactivate transcription of several promoter-enhancer constructs in transfection experiments (3–6). In addition, transgenic mouse experiments have directly implicated HBV-X in the pathogenesis of hepatocellular carcinoma (7).

The mechanism by which HBV-X functions as a transactivator remains obscure (8), but several diverse models have been proposed. Since HBV-X does not possess a classic DNA-binding motif or DNA-binding activity *in vitro*, it is

thought to interact with host-cell proteins to exert its effects. Alternative models have proposed that HBV-X interacts with cellular transcription factors either as a coactivator (9, 10) or to stabilize DNA–protein binding (11). HBV-X has been reported to possess intrinsic protein kinase activity (12) and, therefore, suggested to regulate transcription indirectly. We sought to test these models and identify the proximal target for HBV-X action. We found that while an AP-1-responsive enhancer may be transactivated by HBV-X, the effect is not due to direct interaction of HBV-X and AP-1. Rather, we discovered that HBV-X acts through cellular signaling pathways involving the serine/threonine kinases, protein kinase C (PKC), and Raf-1. Therefore, HBV-X has a peripheral site of action and regulates transcription indirectly, by perturbing the activity of host-cell signal transduction pathways. This mechanism could provide a basis for the promiscuous nature of HBV-X transactivation. In addition, it suggests that HBV-X may affect other cellular processes besides transcription, which may explain the role of HBV-X in the pathogenesis of liver tumors.

MATERIALS AND METHODS

Expression Plasmids. The HBV-X expression construct was generated from adw2 subtype HBV DNA. The *Nco*I–*Bgl*II fragment of HBV DNA (nt 1376–1987) was cloned into the plasmid pCMVPLPAΔS (from David Strandberg, University of California–San Francisco; UCSF), which contains the cytomegalovirus (CMV) immediate early gene 1 (*IE1*) enhancer-promoter. Expression plasmids for PKC (α subtype; from Nachman Mazurek, Biomembrane Institute, Seattle) (13), Raf-1, and Raf-C4 mutant (from Ulf Rapp, National Cancer Institute, Frederick, MD) (14) have been described. A transcription vector for *c-jun* was provided by Jeff Johnson (UCSF) and an expression vector for *c-jun* (pRSVcjun) was provided by Michael Blonar (UCSF).

Reporter Plasmids. pCMV-CAT was constructed by ligating the *Pvu*II–*Hind*III chloramphenicol acetyltransferase (CAT) gene fragment of pSV2CAT (15) into *Pvu*II–*Hind*III-digested pCMVPLPAΔS. The remaining CAT reporter plasmids have been described: pTE2ΔSN (16) with the HSV thymidine kinase (tk) promoter; pEN-CAT, pEN-preC-CAT, preC-CAT, and preS1-CAT and preS2-CAT, with the HBV enhancer-X promoter, enhancer-core promoter, core promoter, preS1 promoter, and preS2 promoter, respectively (17); pβ-CAT (18) with the rat β-actin promoter; pMET-CAT

Abbreviations: HBV, hepatitis B virus; PKC, protein kinase C; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase; HTLV-I, human T-cell leukemia virus type I; LTR, long terminal repeat; HIV, human immunodeficiency virus; SV40, simian virus 40; FCS, fetal calf serum; RSV, Rous sarcoma virus.

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(from G. M. Gorman, Genentech) with 2 kb of mouse metallothionein I 5' flanking sequence; pHTLV-I-CAT (19) with the human T-cell leukemia virus type I (HTLV-I) U3 and 105 bp of the U5 region of the long terminal repeat (LTR) and pTAR-1-CAT (20) with the human immunodeficiency virus (HIV) LTR (from Matija Peterlin, UCSF); pMCAT and pAP1CAT (10) bearing a simple promoter alone or in combination with an AP-1 minienhancer, respectively (from Ben Yen, UCSF). The luciferase reporter plasmids pRSV-L, pSV232AL-AΔ5' [with the enhancerless simian virus 40 (SV40) early promoter] (21), and pPRL7s-luc with seven repeats of the exocrine pancreatic enhancer core (22) upstream of a truncated rat prolactin promoter (from Chris Nelson, University of California, Riverside) have been described.

Transfections. Cells were cultured as follows: CV-1, COS-7, F9, and Ltk⁻ cells, DME-H16 (Dulbecco's modified Eagle's medium low glucose, GIBCO/BRL)/10% (vol/vol) fetal calf serum (FCS); HepG2, HeLa, and AR4-2J cells, DME-H21 (Dulbecco's modified Eagle's medium high glucose, GIBCO/BRL)/10% FCS; HIT-T15 M2.2.2 cells, DME-H16/2.5% FCS/12.5% (vol/vol) horse serum; CHO cells, Ham's F12/10% FCS; Jurkat cells, RPMI 1640 medium/10% FCS. All cell lines and media were supplied by the UCSF Cell Culture Facility. Jurkat cells were transfected using DEAE-dextran (23) whereas other cells were transfected by the calcium phosphate coprecipitation procedure (24).

At the end of the experiment, cells were washed with phosphate-buffered saline and resuspended in 250 mM Tris-HCl (pH 7.8) for CAT assays or 100 mM potassium phosphate (pH 7.8) for luciferase assays and were lysed by three freeze-thaw cycles. CAT assays were quantitated by TLC followed by liquid scintillation of the excised spots or by extraction of reaction products with ethyl acetate (24). Luciferase activity was measured using a luminometer from Analytical Luminescence Laboratory (San Diego). All experiments were repeated at least three times.

Recombinant HBV-X and Antiserum. Recombinant HBV-X was produced using the *Escherichia coli* expression vector described by Blanas and Rutter (25). The HBV-X open reading frame was PCR-amplified and cloned downstream of the FLAG epitope and heart muscle kinase recognition sequence. Fusion protein was produced in BL21 cells and purified from inclusion bodies by mild detergent washing (26). This yielded a protein preparation that was ≈95% pure. For biochemical analyses, the crude protein was denatured in 8 M urea and refolded by dialysis into 25 mM acetic acid (12). A rabbit antiserum was generated that was able to immunoprecipitate a 17-kDa protein from *Xenopus* oocytes injected with HBV-X mRNA (data not shown).

In Vitro Translation and Immunoprecipitation. HBV-X and c-Jun were produced by *in vitro* transcription and translation using kits from Promega. Immunoprecipitations were performed in a low-stringency buffer (50 mM Tris-HCl, pH 7.5/190 mM NaCl/6 mM EDTA/1% Triton X-100) to maximize protein-protein interactions (27). Antigens were incubated with antisera against the FLAG M2 epitope (Kodak-IBI), c-Jun (antiserum provided by Jeff Miner, UCSF), or HBV-X overnight at 4°C. Antigen-antibody complexes were recovered using Pansorbin (Calbiochem), and antigens were released by boiling with Laemmli sample buffer and analyzed by SDS/PAGE on 15% gels.

Electrophoretic-Gel-Mobility-Shift Assay. Complementary oligonucleotides representing the collagenase AP-1 site (5'-GATCCGCGCTGAGTCAC-3' and 5'-GATCGTGACTCAGCGCG-3') were labeled with polynucleotide kinase and [γ -³²P]ATP and annealed. CV-1 cell nuclear extract (28) (5 μg) or *in vitro* translation mixture (1 μl) was added to AP-1 binding reaction mixtures (29), which were analyzed on 0.5× TBE/polyacrylamide gels (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3). HBV-X was preincubated with the AP-1 for 15 min on ice prior to the addition of probe.

RESULTS

HBV-X Protein Is a Promiscuous Transactivator. HBV-X was expressed from a CMV expression vector in cotransfection experiments with various reporter genes. HBV-X activates transcription at very low concentrations and high concentrations of HBV-X inhibit transcription of the reporter gene. In CV-1 cells for example, the activity of the Rous sarcoma virus (RSV) LTR showed a dramatic biphasic response to increasing levels of HBV-X expression (3.7-, 4.3-, 4.8-, 3.2-, and 0.4-fold transactivation in response to 0.02, 0.08, 0.4, 2, and 10 μg of pCMV-X, respectively). Introduction of a frameshift (+1) after codon 10 in the HBV-X open reading frame or deletion of the 5' half of the open reading frame eliminates the ability of HBV-X to transactivate (data not shown).

In an effort to understand the mechanism by which HBV-X exerts its effects, we determined whether the transactivation was restricted to certain classes of promoters. Experiments were performed in CV-1, Jurkat, AR42J, HepG2, HIT, COS, and L cells, testing 13 promoter-enhancer constructs derived from both viral and cellular genes. Some of the results are shown in Fig. 1. HBV-X transactivated all reporter constructs tested, with the exception of the HTLV-I LTR, but the degree of transactivation was cell-type dependent. For example, the RSV LTR was highly induced in CV-1, Jurkat, and AR42J cells but was not or was minimally transactivated

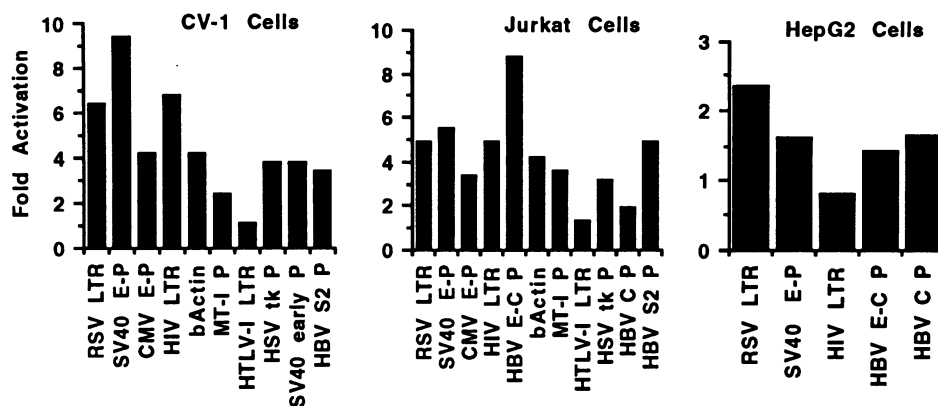


FIG. 1. HBV-X is a promiscuous transactivator. Cells were transfected in the presence and absence of pCMV-X using the various promoter constructs indicated. HSV, herpes simplex virus; E-P, enhancer-promoter; bActin, β -actin; MT-I, metallothionein I; tk, thymidine kinase; E-C, enhancer-core.

in L, HepG2, COS, and HIT cells. In general, the response of the promoters to HBV-X appeared to be much lower in the latter group of cells. We confirmed that HBV-X was made in these cells by immunoprecipitation (data not shown). Thus, the reason for the lack of response to HBV-X in some cell types is unclear. Since the response to HBV-X shows a distinct concentration-dependent optimum, it is possible that different cells show variable sensitivity to HBV-X.

HBV-X Activates but Does Not Associate with the Enhancer or its Binding Proteins. It is clear that HBV-X transactivates a variety of promoter-enhancer structures that possess unrelated regulatory elements. This promiscuous activity suggests that the target of HBV-X is not a specific DNA element or protein bound to this element. However, in several experiments it appeared that promoter-enhancer combinations were activated more than promoters alone. For example, the HBV core protein promoter was transactivated only ≈ 2 -fold, whereas the core promoter adjacent to the HBV enhancer I was activated 9-fold in Jurkat cells (Fig. 1). To test whether transactivation by HBV-X is dependent on functional enhancer elements, a promoter that contains only a "TATA box" and Sp1 site (pMCAT) was tested in F9 cells. This promoter was not activated by cotransfection with HBV-X. In contrast, when three tandem AP-1 binding sites (AP-1 minienhancer) were inserted upstream of the simple promoter (pAP1CAT), HBV-X was able to transactivate (Fig. 2). This activation was dependent on cotransfection of a *c-jun* expression vector since undifferentiated F9 cells possess low endogenous AP-1 activity (30). To rule out the possibility that HBV-X simply increased basal AP-1 levels, a titration experiment was performed. Increasing amounts of *c-jun* expression vector produced a dose-dependent increase in transcription, which reached saturation at high plasmid concentrations. Even at saturating concentrations of *c-Jun*, HBV-X was still able to transactivate (data not shown). These data suggest that HBV-X acts synergistically with *c-Jun* rather than simply increasing its effective concentration.

To investigate whether HBV-X associates directly with *c-Jun*, the proteins were made by *in vitro* translation and immunoprecipitated with antisera against *c-Jun* or HBV-X (Fig. 3), under conditions that allow *c-Jun* to associate with Fos (31). The antisera did not precipitate the respective heterologous proteins whether the proteins were mixed after translation or were cotranslated. In another attempt to detect association of HBV-X and AP-1, recombinant HBV-X was added to DNA binding reaction mixtures containing AP-1 in CV-1 cell nuclear extract (Fig. 3) or in reticulocyte lysate

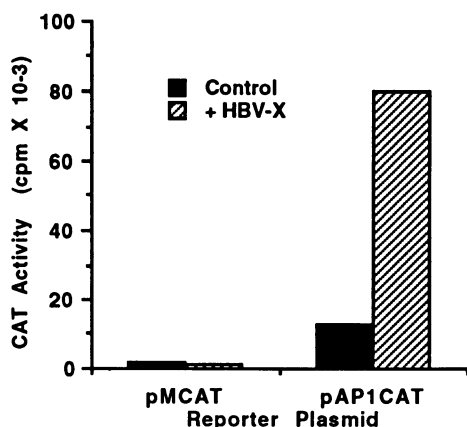


FIG. 2. Transactivation by HBV-X is dependent on enhancer elements. F9 cells were transfected with a minimal promoter construct alone (pMCAT) or with an AP-1 minienhancer (pAP1CAT). Cells were also transfected with pRSVcjun to increase endogenous AP-1 activity.

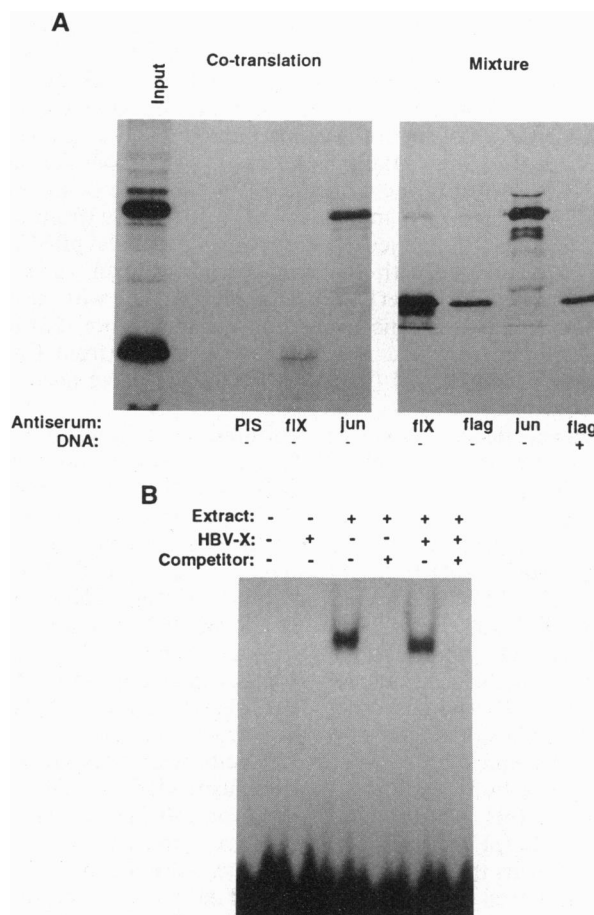


FIG. 3. HBV-X and *c-Jun* do not interact *in vitro*. (A) HBV-X and *c-Jun* were produced by *in vitro* translation in the presence of [³⁵S]methionine, either separately and then mixed (Right) or by cotranslation of mRNAs (Left) and then immunoprecipitated with preimmune serum (PIS) or antisera against HBV-X (flX), *c-Jun*, or the FLAG M2 epitope (flag). Both native HBV-X (17 kDa) (Left) and HBV-X with the N-terminal FLAG epitope (19 kDa) (Right) were used. Immunoprecipitation was also attempted in the presence of unlabeled oligonucleotides representing an AP-1 binding site. (B) Electrophoretic-gel-mobility-shift assays of AP-1 activity in CV-1 cells alone or in the presence of recombinant HBV-X.

programmed with *c-jun* RNA (data not shown). HBV-X failed to alter AP-1 binding, even when significant (up to 200 ng) amounts of protein were added. Larger amounts were not practical due to limitations in HBV-X solubility. HBV-X did not affect the size or affinity of the AP-1 complex. Kinetic parameters such as AP-1 "on rate" or "off rate" were similarly unaffected (data not shown).

We could not rule out the possibility that the hypothetical interaction between HBV-X and AP-1 is weak and, therefore, undetectable *in vitro*. To circumvent this problem, we attempted to detect HBV-X and AP-1 association *in vivo*. The inhibition of promoter function observed at high concentration of HBV-X has been suggested to result from the association of HBV-X with some component of the transcription complex or "squenching" (32). We, therefore, tested whether HBV-X could squelch the ability of *c-Jun* to activate transcription from the AP-1-dependent promoter in F9 cells. The response of the AP-1-dependent promoter to an increment of *c-Jun* was measured alone or in the presence of an optimal dose or a 100-fold excess of HBV-X expression plasmid. While high levels of HBV-X did not transactivate as well as low levels, they did not inhibit activation in response to *c-Jun* (data not shown). Thus, HBV-X did not squelch *c-Jun*-activated transcription. We also tested whether HBV-X

possesses an "interaction domain" that allows it to bind to c-Jun or some other component of the transcription initiation complex, by fusing the activation domain from herpes simplex virus VP-16 to the N terminus of HBV-X. Transactivation by the fusion protein was equivalent to that observed for native HBV-X over a similar dose range and in different cell lines (data not shown), suggesting that HBV-X is not tethered to the transcription complex.

Transactivation by HBV-X Is Dependent on Cellular Kinases. HBV-X has been reported to possess protein kinase activity (12), an observation that was surprising since the HBV-X structure does not possess either hallmark kinase or ATP-binding domains. We have not detected intrinsic autophosphorylation or histone kinase activity associated with recombinant HBV-X produced in *E. coli* or *Xenopus* oocytes. Nonetheless, HBV-X possesses recognition motifs for several cellular kinases including cAMP-dependent protein kinase (aa 28–31, 72–75, and 78–81), casein kinase (aa 105–107), histone kinase (aa 28–31 and 72–75), PKC (aa 54–56 and 75–77), and proline-dependent kinase (cdc2; aa 39–40 and 40–41). Therefore, it is possible that the kinase activity attributed to HBV-X was due to contaminating kinases that utilize HBV-X as a substrate.

The activity of several transcription factors is regulated by phosphorylation (33). To determine whether cellular protein kinases might regulate the activity of HBV-X, transactivation assays were performed in serum-starved cells. Interestingly, the ability of HBV-X to transactivate the SV40 early promoter or AP-1 minienhancer was diminished when cells were grown in 0.5% serum (Table 1). This result suggests that signaling pathways activated by growth factors are required for HBV-X action. Several growth factors are thought to act through serine/threonine kinases including PKC, Raf-1, and mitogen-activated protein kinase. To determine whether these kinases are required for HBV-X action, kinase inhibition studies were performed. HBV-X transactivation of the SV40 early promoter (Table 1), and the AP-1 minienhancer (data not shown), was blocked by phorbol ester-induced down-regulation of PKC. Transactivation was also blocked by the kinase inhibitor staurosporine; the low concentrations of inhibitor that were used may be specific to inhibition of PKC (34). Importantly, staurosporine had no effect on basal expression but only inhibited the effect of HBV-X.

Table 1. Function of HBV-X is controlled by PKC and Raf-1 kinase activities

Experiment	CAT activity, cpm		Fold activation
	Basal	+ X	
1. pAP1CAT in HeLa cells			
10% FCS	607	2,375	3.9
0.5% FCS	364	175	0.5
2. pSV2CAT in CV-1 cells			
Control	7,334	54,447	7.4
PMA	6,012	10,418	1.7
3. pSV2CAT in CV-1 cells			
Control	14,103	165,552	11.7
Staurosporine (0.1 nM)	17,945	36,983	2.1
Staurosporine (1.0 nM)	12,749	14,325	1.1
4. pAP1CAT in F9 cells			
Control	7,093	18,494	2.6
Raf-C4	9,514	9,189	1.0

Cells were transfected with the indicated reporter plasmids in the absence or presence of pCMV-X and treated as follows. Experiments: 1, 10% or 0.5% FCS beginning 4 h prior to transfection; 2, 100 nM phorbol 12-myristate 13-acetate (PMA) beginning 2 h prior to transfection; 3, staurosporine beginning 2 h prior to transfection; 4, cells were transfected with or without an expression vector for the Raf-C4 dominant negative mutant.

Raf-1 is a mitogen-activated kinase that may link activated growth factor receptors and Ras with the mitogen-activated protein kinase cascade (35, 36). A deletion mutant of Raf-1 kinase (Raf-C4) has a dominant negative effect in transfected cells and has been used to show that Ras activates Raf-1 (14). Expression of this mutant blocked HBV-X transactivation of the AP-1-dependent promoter (Table 1), even at concentrations that did not affect basal expression. These data indicate that Raf-1, in addition to PKC, is required for HBV-X to transactivate AP-1.

Since both AP-1 and HBV-X are potential substrates for serine/threonine kinases, the reason why PKC and Raf-1 might be required for HBV-X action was ambiguous. Therefore, it was important to determine whether HBV-X is a substrate for PKC and Raf-1 or whether HBV-X acts upstream and activates PKC and Raf-1. PKC and Raf-1 were overexpressed in cells to distinguish between these two possibilities. These kinases each increased the activity of the AP-1-dependent promoter 3- to 5-fold. Whereas HBV-X transactivated 3- to 4-fold under basal conditions, HBV-X was unable to transactivate when PKC or Raf-1 was overexpressed (Fig. 4). These experiments imply that PKC and Raf-1 act downstream of HBV-X. It is unclear whether HBV-X activates PKC and/or Raf-1, however, since expression of HBV-X in *Xenopus* oocytes did not affect membrane-bound or cytosolic histone kinase activity (data not shown).

DISCUSSION

We have used HBV-X-mediated transactivation as a functional assay to define the cellular targets and mode of action of HBV-X. Although the transcription factor AP-1 is activated by HBV-X, we were unable using several types of experiments to detect a direct interaction that would explain the synergism between HBV-X and AP-1. Previous models that suggest that HBV-X binds to transcription factors to coactivate transcription (9, 10) or to stabilize binding to DNA (11) were, therefore, not supported by our studies. It is difficult to reconcile the previous data, but HBV-X has low solubility and tends to form aggregates even at moderate concentrations *in vitro*. Therefore, *in vitro* binding activity of HBV-X should be interpreted with caution. Our data strongly suggest that HBV-X activates transcription indirectly, not by forming part of the transcription complex, but rather by stimulating regulatory pathways that in turn affect transcrip-

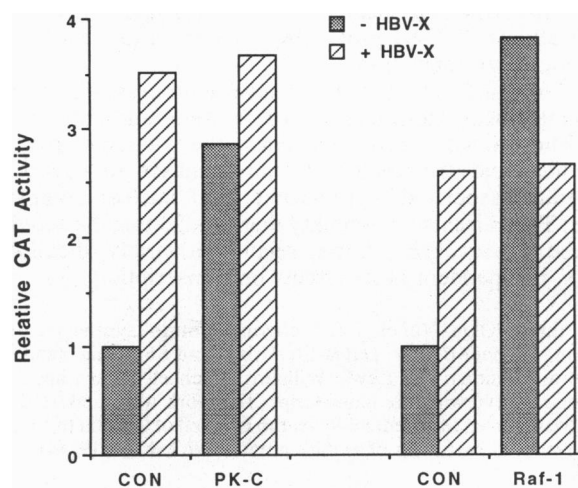


Fig. 4. Expression of PKC and Raf-1 diminishes the ability of HBV-X to transactivate. Activation of the AP-1 minienhancer by HBV-X was measured in F9 cells (also transfected with pRSVcjun) in the absence (CON) or presence of cotransfected expression plasmids for PKC or Raf-1 (1 μ g of each).

tion factor activity. Specifically, HBV-X acts upstream of PKC and Raf-1 kinase in a signaling cascade.

While the finding that HBV-X acts in a signaling pathway is important, the specific mechanism of action is unclear. The protein has interesting structural properties that likely reflect its action, however. The N-terminal half of HBV-X is rich in serine and threonine and possesses consensus kinase recognition sequences for cAMP-dependent protein kinase, PKC, histone, casein kinases, and proline-directed kinase (cdc2). HBV-X appears to be phosphorylated *in vivo* (37), although the effect of phosphorylation on HBV-X activity is unknown. Immunolocalization and cell fractionation (37) studies indicate that HBV-X is found in the cytoplasm and nucleus, associated with cytoskeletal and membrane fractions. Finally, HBV-X has an extremely short half-life *in vivo* (ref. 37; J.C.C., unpublished observations), which explains its low steady-state levels in cells.

Our experiments clearly implicate PKC and Raf-1 kinase in mediating HBV-X action. A recent report by Kekule *et al.* (38) also implicates PKC as an effector of HBV-X and suggests that HBV-X directly activates PKC. Though this result is generally consistent with our studies, the findings differ in two significant respects. First, we have demonstrated that Raf-1 kinase, in addition to PKC, is a potential effector in the HBV-X response pathway. This difference is significant since others have reported that inhibition of PKC does not affect the ability of HBV-X to transactivate NF- κ B, at least in hepatocytes (39). It is not clear whether Raf-1 and PKC interact, although it has been suggested that PKC can activate Raf-1 (36), which in turn probably activates the mitogen-activated protein kinase cascade (35, 36).

A second difference is that we have found no evidence that HBV-X can directly activate PKC, a finding that has been supported by others (39). Moreover, we observed that the ability of HBV-X to transactivate was ablated in serum-starved cells. This implies that growth factor stimulation is required for HBV-X action and, hence, that HBV-X alone cannot activate PKC and Raf-1. Therefore, HBV-X may regulate the coupling of growth factor receptors to signaling cascades, perhaps by affecting kinase or phosphatase activities. Alternatively, HBV-X may alter the turnover of PKC and/or Raf-1 kinase activity after normal activation. Inactive PKC is free in the cytoplasm but associates with membrane receptors after activation, presumably allowing it to interact with membrane-associated substrates (40). Proteolysis leads to down-regulation of PKC that follows normal activation (40). Since HBV-X associates with membranes, it is possible that it affects PKC by prolonging membrane association or reducing down-regulation.

PKC (40) and Raf-1 (35) affect several processes including cell metabolism, morphology, proliferation, and differentiation. These diverse activities reflect the wide spectrum of substrates and the ability of these kinases to reside in membrane-associated, cytoplasmic, and nuclear compartments. This raises the possibility that by affecting the activity of these kinases, HBV-X may regulate a variety of cellular events, independent of its effects on transcription.

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- Standring, D. N. & Rutter, W. J. (1986) in *Progress in Liver Disease*, eds. Popper, H. & Schaffner, F. (Grune & Stratton, Orlando, FL), Vol. 8, pp. 311–333.
- Ganem, D. & Varmus, H. E. (1987) *Annu. Rev. Biochem.* **56**, 654–693.
- Twu, J.-S. & Robinson, W. S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2046–2050.
- Spandau, D. F. & Lee, C.-H. (1988) *J. Virol.* **62**, 427–434.
- Seto, E., Yen, T. S. B., Peterlin, B. M. & Ou, J. H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8286–8290.
- Siddiqui, A., Gaynor, R., Srinivasan, A., Mapoles, J. & Farr, R. W. (1989) *Virology* **169**, 479–484.
- Kim, C.-M., Koike, K., Saito, I., Miyamura, T. & Jay, G. (1991) *Nature (London)* **351**, 317–320.
- Will, H. (1991) *J. Hepatol.* **13**, Suppl. 4, S56–S57.
- Unger, T. & Shaul, Y. (1990) *EMBO J.* **9**, 1889–1895.
- Seto, E., Mitchell, P. J. & Yen, T. S. (1989) *Nature (London)* **344**, 72–74.
- Maguire, H. F., Hoeffler, J. P. & Siddiqui, A. (1991) *Science* **252**, 842–844.
- Wu, J. Y., Zhou, Z.-Y., Judd, A., Cartwright, C. A. & Robinson, W. S. (1990) *Cell* **63**, 687–695.
- Megidish, T. & Mazurek, N. (1989) *Nature (London)* **342**, 807–811.
- Bruder, J. T., Heidecker, G. & Rapp, U. R. (1992) *Genes Dev.* **6**, 545–556.
- Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051.
- Edlund, T., Walker, M. D., Bar, P. J. & Rutter, W. J. (1985) *Science* **230**, 912–916.
- Antonucci, T. K. & Rutter, W. J. (1989) *J. Virol.* **63**, 579–583.
- Melloul, D., Aloni, B., Calvo, J., Yaffe, D. & Nudel, U. (1984) *EMBO J.* **3**, 983–990.
- Sodroski, J. G., Rosen, C. A. & Haseltine, W. A. (1984) *Science* **225**, 381–385.
- Tong-Starksen, S. E., Luciw, P. A. & Peterlin, B. M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6845–6849.
- de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R. & Subramani, S. (1987) *Mol. Cell. Biol.* **7**, 725–737.
- Meister, A., Weinrich, S. L., Nelson, C. & Rutter, W. J. (1989) *J. Biol. Chem.* **264**, 20744–20751.
- Durand, D. B., Bush, M. R., Morgan, J. G., Weiss, A. & Crabtree, G. R. (1987) *J. Exp. Med.* **165**, 395–407.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
- Blonar, M. A. & Rutter, W. J. (1992) *Science* **256**, 1014–1018.
- Bohmann, D. & Tjian, R. (1989) *Cell* **59**, 709–717.
- Anderson, D. J. & Blobel, G. (1983) *Methods Enzymol.* **96**, 111–120.
- Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
- Yang-Yen, H. F., Chambard, J.-C., Sun, Y.-L., Smeal, T., Schmidt, T. J., Drouin, J. & Karin, M. (1990) *Cell* **62**, 1205–1215.
- Yang-Yen, H.-F., Chiu, R. & Karin, M. (1990) *New Biol.* **2**, 351–361.
- Hai, T. & Curran, T. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3720–3724.
- Ptashne, M. (1988) *Nature (London)* **335**, 683–689.
- Hunter, T. & Karin, M. (1992) *Cell* **70**, 375–387.
- Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. & Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* **135**, 397–402.
- Rapp, U. R. (1991) *Oncogene* **6**, 495–500.
- Pelech, S. L. & Sanghera, J. S. (1992) *Science* **257**, 1355–1356.
- Schek, N., Bartenschlager, R. & Schaller, H. (1991) *Oncogene* **6**, 1735–1744.
- Kekule, A. S., Lauer, U., Weiss, L., Luber, B. & Hofschneider, P. H. (1993) *Nature (London)* **361**, 742–745.
- Lucito, R. & Schneider, R. J. (1992) *J. Virol.* **66**, 983–991.
- Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. (1987) *Annu. Rev. Biochem.* **56**, 567–613.